Volume 310, number 3, 216–218 FEBS 11596 October 1992

A mutant α-amylase with enhanced activity specific for short substrates

Ikuo Matsui[a], Kazuhiko Ishikawa[a], Sachio Miyairi[a], Sakuzo Fukui[b] and Koichi Honda[a]

[a]*National Chemical Laboratory for Industry, Tsukuba, Ibaraki 305, Japan* and [b]*Department of Engineering, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan*

Received 23 July 1992; revised version received 17 August 1992

The 210th lysine (K210) at the active site in *Saccharomycopsis fibuligera* α-amylase was altered to arginine (R) or asparagine (N) by site-directed mutagenesis. Replacement of K210 by R strengthened the 7th and weakened the 8th subsite affinities. K210 was found to contribute to both the 8th and the 7th subsites. The catalytic activity of the K210R enzyme for the hydrolysis of maltose (G_2) was three-times higher than that of the native enzyme due to an increase in the affinity of the 7th subsite adjacent to the catalytic site, whereas the activity of the K210N enzyme for G_2 was decreased to 1% of that of the native enzyme by a reduction in the 7th subsite affinity.

α-Amylase; Subsite affinity; Site-directed mutagenesis; Maltooligosaccharide; *Saccharomycopsis*

1. INTRODUCTION

The steric structure of α-amylase binding maltooligosaccharide in a productive form has not been clarified because of the difficulty of isolating the enzyme–substrate (ES) complex. Matsuura et al. proposed a substrate binding model for fitting the maltoheptaose (G_7) molecule to the active cleft of the Taka-amylase (TAA) molecule [1]. The authors have also proposed a substrate-binding model for *Saccharomycopsis fibuligera* α-amylase (Sfamy) [2] involving a modification of the TAA model on the basis of the steric structure predicted theoretically and on the basis of the subsite structure of Sfamy [3,4]. Analysis of the kinetic parameters and subsite affinities of various mutant enzymes generated by substitution of the amino acid residues that are the possible components of subsites might clarify the role of these residues in recognizing the substrate. We prepared mutant enzymes carrying a substitution of the 210th lysine (K210), which is one of the possible components of a major subsite (the 8th subsite) [4], and analyzed their enzymic characteristics. We found short substrate-specific enhancement in the catalytic activity of the mutant enzyme (K210R). The present paper describes the properties of the mutant enzymes (K210R and K210N) and the contribution of K210 to both the 7th and 8th subsite affinities.

Correspondence address: I. Matsui, National Chemical Laboratory for Industry, Tsukuba, Ibaraki 305, Japan. Fax: (81) (298) 544 487.

Abbreviations: Sfamy, *Saccharomycopsis fibuligera* α-amylase; G_2, maltose; G_3•, G_4• and G_5•, reducing end-labeled maltotriose, maltotetraose and maltopentaose.

2. MATERIALS AND METHODS

2.1. *Construction of the mutated genes and purification of the wild-type and mutant Sfamy's*

An *Eco*RI–*Pst*I DNA fragment (2.5 kb) containing the Sfamy gene was isolated from the plasmid, pSfα 1 [5,6] and subcloned into the M13 phage vector. Replacement of K210 with R and N was carried out using synthetic oligonucleotides according to the method described by Kunkel [7]. The wild-type and mutated genes were inserted into the multicloning site of the vector, YEp351 [8]. *Saccharomyces cerevisiae* strain KK4 cells were transformed with the constructed plasmids according to the method described by Hinnen et al. [9]. The transformant cells were cultured in a YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose) at 30°C for 6 days. From these culture filtrates, the secreted wild-type and mutant Sfamy enzymes were purified by chromatography using DE52–cellulose (Whatman), Superose 12 (Pharmacia), and butyl-Toyopearl 650S columns [2]. The purified enzyme sample gave a single protein band after SDS-polyacrylamide gel electrophoresis.

2.2. *Analysis of the product from reducing end-labeled maltotriose (G_3•), maltotetraose (G_4•) and maltoheptaose (G_5•)*

Reducing end-labeled maltooligosaccharides were prepared according to the method described by Matsui et al. [4] and Kobayashi et al. [10]. The bond-cleavage patterns and the kinetic parameters (k_{cat}/K_m, where k_{cat} and K_m represent the molecular activity and the Michaelis constant, respectively) of the mutant and wild-type enzymes for G_3•, G_4•, and G_5•, were determined by radioactive analysis [4]. The enzymic reaction was performed at 25°C in a 420 μl of 25 mM acetate buffer solution (pH 5.25). The reaction products were separated by paper chromatography and their respective radioactivities were counted with a liquid scintillation counter [11].

2.3. *Evaluation of the subsite affinities of K210R, K210N, and wild-type enzymes*

On the basis of the subsite theory [12], Suganuma et al. devised a simple method to evaluate subsite affinities by using a series of end-labeled substrates [11]. The subsite affinities of the mutant and wild-type enzymes were evaluated by this method.

Published by Elsevier Science Publishers B.V.

2.4. *Determination of the kinetic parameters for the hydrolysis of maltose (G_2) and Amylose A and evaluation of the summed affinities of the 6th and 7th subsites*

The reaction mixtures of G_2 and the enzyme in a 50 mM acetate buffer (pH 5.25) were incubated at 25°C and the glucose produced was analyzed with a Glucose c-Test kit (Wako Pure Chemicals). The hydrolysis reaction of Amylose A (M_r = ca. 2,900, Nakarai Chemicals) was done in a 50 mM acetate buffer (pH 5.25) at 25°C and was stopped with the addition of 0.1 N NaOH solution. The reducing power of the hydrolyzate was monitored using the Somogy–Nelson method [13]. Initial velocities were obtained directly from the initial slopes of the time-course plots. The K_m and k_{cat} values were calculated using the least squares method [14] and the Michaelis–Menten equation. The summed affinities of the two subsites adjacent to the catalytic site ($A_6 + A_7$) of the mutant and wild-type enzymes were evaluated in the manner described by Matsui et al. [4] and Isawa et al. [15].

3. RESULTS AND DISCUSSION

Table I indicates that the mutations, K210R and K210N, increase cleavage frequency at a specific bond of G_3 (the 1st bond from the reducing end side), and cause a shift of the major cleavage site of G_4 from the 2nd to the 1st bond from the reducing end side.

Table II lists the kinetic parameters of the mutant and wild-type enzymes. Compared to the wild-type enzyme, the k_{cat}/K_m values of K210R, for the short substrates such as G_2 and G_3, exhibit a two- to three-fold increase, while the same values for G_4, G_5, and Amylose A($\sim G_{18}$) decrease to 20–50%. In contrast, the k_{cat}/K_m values of K210N, for substrates shorter than G_5, decrease to less than a few % of those of the wild-type enzyme.

Table III shows the 4th, 5th, and 8th subsite affinities (A_4, A_5, and A_8) and the summed affinities for the 6th and 7th subsites adjacent to the catalytic site ($A_6 + A_7$) of the mutant and wild-type enzymes. Fig. 1 illustrates these affinities as histograms. The mutations decreased affinities, A_4, A_5, and A_8. The 8th affinity (A_8) was lowered the most. The effect of the K210R mutation on $A_6 + A_7$ was opposite to that of the K210N: K210R raised $A_6 + A_7$ and K210N lowered $A_6 + A_7$.

As reported previously [3], the Sfamy protein is composed of a main (M) domain and a C-terminal (C) domain, where the M domain is folded into a $(\alpha/\beta)_8$ barrel structure. The K210 is located on a loop called L_4, which is one of the eight loops comprising the M domain. The 211th histidine (H211), which is a possible residue composing the 7th subsite, is also present on the L_4 loop. One of the catalytic residue candidates, the 270th aspartic acid (D207), is present between the C-terminal end of a β-sheet called βA_4 and the N-terminal end of the L_4 loop. As K210 is placed near D207 and H211 on the same loop, the intrinsic rate constant (k_{int}) value for the hydrolysis and the 7th subsite affinity might be influenced by the mutation at the 210th position. According to the subsite theory [12], the k_{int} value, which is independent of the chain length of the substrate and the binding mode, is identical to the k_{cat} value if the substrate is larger than the substrate binding site. In fact, the mutation decreases the k_{int} values which are identical to the k_{cat} values for Amylose A, as shown in Table II. However, the mutation might not affect the 6th subsite affinity, since the 210 residue on the L_4 loop

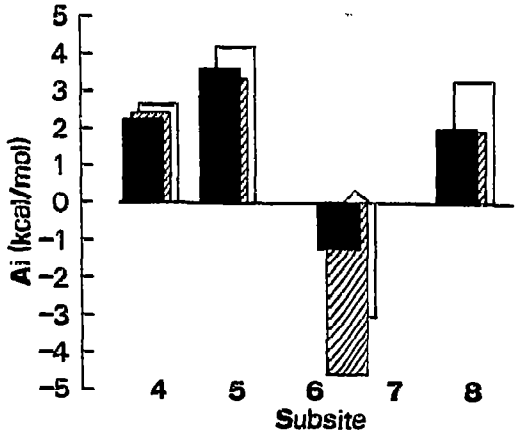

Fig. 1. Histograms showing the subsite affinities (A_i) of the mutant and wild-type enzymes. The wedge represents the catalytic site. The columns indicate the affinities of the K210R (filled bars), K210N (hatched bars) and wild-type (open bars) enzymes.

Table I

Bond-cleavage patterns for the hydrolysis of end-labeled G_3 and G_4 catalyzed by the K210R, K210N and wild-type enzymes at pH 5.25 and 25°C

	G_3			G_4
	$[E]_0$ (M)	$[S]_0$ (M)	Frequency[a]	Frequency[a]
K210R	4.9×10^{-7}	1.9×10^{-5}	G—G—G*	G—G—G—G*
			0.063 0.937	0.407 0.593
K210N	1.5×10^{-6}	1.9×10^{-5}	G—G—G*	G—G—G—G*
			0.084 0.916	0.120 0.266 0.614
Wild-type	7.3×10^{-7}	1.9×10^{-5}	G—G—G*	G—G—G—G*
			0.176 0.824	0.745 0.255

$[E]_0$ and $[S]_0$ represent the concentrations of the substrate and enzyme, respectively.
[a] Numbers indicate the normalized cleavage frequencies of the indicated bonds.
*Radioactive glucose residue.

Table II

Kinetic parameters for the hydrolysis of maltooligosaccharides catalyzed by the K210R, K210N and wild-type enzymes at pH 5.25 and 25°C

n[a]	k_{cat} (min^{-1})			K_m (M)			k_{cat}/K_m ($min^{-1} \cdot M^{-1}$)		
	K210R	K210N	Wild-type	K210R	K210N	Wild-type	K210R	K210N	Wild-type
2	7.9×10^{-2} (172)[b]	2.8×10^{-3} (6)	4.6×10^{-2} (100)	5.1×10^{-2}	4.5×10^{-1}	8.6×10^{-2}	1.5 (283)	6.2×10^{-3} (1)	5.3×10^{-1} (100)
3	–[c]	–	–	–	–	–	1.2×10^{4} (211)	1.9×10^{2} (3)	5.7×10^{3} (100)
4	–	–	–	–	–	–	8.4×10^{5} (49)	1.7×10^{4} (1)	1.7×10^{6} (100)
5	–	–	–	–	–	–	3.0×10^{6} (20)	4.5×10^{5} (3)	1.5×10^{7} (100)
18	6.4×10^{2} (13)	7.6×10^{2} (16)	4.9×10^{3} (100)	3.3×10^{-5}	1.2×10^{-4}	1.1×10^{-4}	1.9×10^{7} (43)	6.6×10^{6} (15)	4.4×10^{7} (100)

[a] Degree of polymerization of the substrate expressed as glucose units.
[b] Percentage based on value of wild-type enzyme.
[c] Value not determined.

is distant from H123 and H297 on the L_3 and L_7 loops which are presumed to be the major components of the 6th subsite [2,3]. Therefore, the change in the summed affinities of the 6th and 7th subsites caused by the replacement is actually due to the change in the 7th subsite affinity, although evaluation of the separate affinities of the 6th and 7th subsites is not possible because the catalytic residues are located between them. Thus, the influence of the 210th residue can be restricted to the 7th and 8th subsite affinities. Replacement of the K210 residue by an N residue, which has a shorter and neutral side chain, decreases the affinities of the 8th and 7th subsites. Substitution of the residue by R, which possess a longer and positively charged side chain, similarly lowers the 8th subsite affinity, but increases the affinity of the 7th subsite. These results indicate that K210 in the native enzyme contributes to both the 7th and 8th subsite affinities.

In conclusion, it has been shown that for Sfamy the enhanced catalytic activity of K210R specific for short substrates, such as G_2 and G_3, is attributable to a substantial increase in the affinity of the 7th subsite adjacent to the catalytic site. Additionally, it has been shown that the decreased catalytic activity of K210N specific for substrates shorter than G_5 ($G_{\leq 5}$) is achieved by a substantial decrease in the 7th and 8th subsite affinities. This finding provides a guiding principle for the comprehension of the substrate recognition mechanism and a means to improve the substrate and product specificities of the enzymes.

Table III

Subsite affinities (A_i) of the K210R, K210N and wild-type enzymes at pH 5.25 and 25°C

A_i	Values of A_i (kcal/mol)		
	K210R	K210N	Wild-type
A_4	2.26	2.42	2.67
A_5	3.63	3.34	4.21
$A_6 + A_7$	−1.19	−4.56	−3.02
A_8	2.03	1.93	3.30

The catalytic site exists between the 6th and 7th subsites.

REFERENCES

[1] Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) J. Biochem. 95, 697–702.
[2] Matsui, I., Ishikawa, K., Miyairi, S., Fukui, S. and Honda, K. (1992) Biochemistry 31, 5232–5236.
[3] Matsui, I., Matsui, E., Ishikawa, K., Miyairi, S. and Honda, K. (1990) Agric. Biol. Chem. 54, 2009–2015.
[4] Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S. and Honda, K. (1991) J. Biochem. 109, 566–569.
[5] Yamashita, I., Itoh, T. and Fukui, S. (1985) Agric. Biol. Chem. 49, 3089–3091.
[6] Itoh, T., Yamashita, I. and Fukui, S. (1987) FEBS Lett. 219, 339–342.
[7] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[8] Hill, J.E., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1986) Yeast 2, 163–167.
[9] Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1929–1932.
[10] Kobayashi, S., Kainuma, K. and French, D. (1983) J. Jpn. Soc. Starch Sci. 30, 62–68.
[11] Suganuma, T., Matsuno, R., Ohnishi, M. and Hiromi, K. (1978) J. Biochem. 84, 293–316.
[12] Hiromi, K. (1970) Biochem. Biophys. Res. Commun. 40, 1–6.
[13] Hiromi, K., Takahashi, Y. and Ono, S. (1963) Bull. Chem. Soc. Jpn. 36, 563–570.
[14] Sakada, M. and Hiromi, K. (1976) J. Biochem. 80, 547–555.
[15] Isawa, S., Aoshima, H., Hiromi, K. and Hatano, H. (1974) J. Biochem. 75, 969–978.